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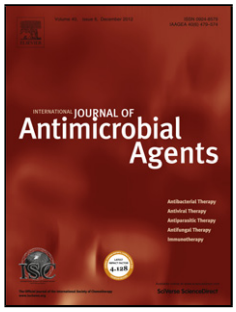

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Deletions in a ribosomal protein-coding gene are associated with tigecycline resistance in *Enterococcus faecium*

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ABSTRACT

Enterococcus faecium is an emerging nosocomial pathogen associated with antibiotic therapy in the hospital environment. Whole-genome sequences were determined for three pairs of related, consecutively collected *E. faecium* clinical isolates to determine putative mechanisms of resistance to tigecycline. The first isolates (1S, 2S and 3S) in each of the three pairs were sensitive to tigecycline [minimum inhibitory concentration (MIC) of 0.125 mg/L]. Following tigecycline therapy, the second isolate in each pair demonstrated increased resistance to tigecycline. Two isolates (1R and 2R) were resistant (MIC of 8 mg/L) and one isolate (3I) demonstrated reduced susceptibility (MIC of 0.5 mg/L). Mutations distinguishing each pair of sensitive and resistant isolates were determined through alignment to a reference genome and variant detection. In addition, a de novo assembly of each isolate genome was constructed to confirm mutations. A total of 16 mutations in eleven coding sequences were determined. Mutations in the *rpsJ* gene, which encodes a structural protein forming part of the 30S ribosomal subunit, were detected in each of the pairs. Mutations were in regions proximal to the predicted tigecycline-binding site. Predicted amino acid substitutions were detected in 1R and 3I. The resistant strains were additionally associated with deletions of 15 nucleotides (2R) and 3 nucleotides (1R). This study confirms that amino acid substitutions in *rpsJ* contribute towards reduced susceptibility to tigecycline and suggests that deletions may be required for tigecycline resistance in *E. faecium*.

1. Introduction

Enterococcus faecium is a nosocomial pathogen associated with antibiotic therapy in the hospital environment that is increasing in prevalence [1,2]. Compared with *Enterococcus faecalis*, which remains the most common *Enterococcus* species in clinical infections, *E. faecium* is intrinsically resistant to a greater number of antibiotics and can efficiently acquire additional antibiotic resistance mechanisms necessitating the use of alternative antibiotics for therapy. Vancomycin resistance is a common and pressing clinical problem in *Enterococcus*. This results from transposon-mediated insertion of genes coding for vancomycin-inactivating enzymes. Tigecycline, a derivative of minocycline, has bacteriostatic activity through binding to the 30S subunit of the bacterial ribosome and inhibiting protein synthesis [2,3]. It provides broad-spectrum activity against multiresistant pathogens such as meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and Enterobacteriaceae with extended-spectrum β-lactamases (ESBLs) or carbapenemases. Tigecycline resistance has been reported [3], mainly in Gram-negative pathogens, e.g. *Acinetobacter baumannii* and *Klebsiella pneumoniae* [4,5], although rarely also in Gram-positive pathogens such as *E. faecalis* and MRSA [6,7]. Tigecycline resistance is most commonly associated with overexpression of efflux pumps, e.g. MarA-mediated overexpression of AcrAB in *Escherichia coli* [3,8].

Whole-genome sequencing (WGS) is a powerful technique that can be used to pinpoint mutations that arise in clinical isolates which have been exposed to antibiotics during treatment. This technique has allowed detailed analysis of pairs of

consecutively isolated tigecycline-susceptible and -resistant isolates of Gram-negative pathogens to be undertaken [5,9]. In this study, WGS was used to investigate the nature of potential mechanism(s) associated with tigecycline resistance and reduced susceptibility in three VRE clinical isolates.

2. Materials and methods

2.1. Bacterial isolates

Two pairs of isolates (1S and 1R, and 3S and 3I) were obtained from patients in a hospital in the West Midlands, UK. Both patients were previous liver transplant recipients and had been diagnosed with hepatic artery thrombosis and intrahepatic collections. A tigecycline-resistant VRE (1R) was isolated from the first patient following a total of 38 days of treatment with tigecycline. The VRE isolate with reduced susceptibility to tigecycline (3I) was isolated following two courses of tigecycline, totalling 43 days of treatment. A third pair consisted of two isolates (2S and 2R), which despite originating from different patients belonged to a persistent endemic clone [determined by pulsed-field gel electrophoresis (PFGE)], isolated in a hospital in Scotland. Isolate 2S was cultured from urine from a renal patient, whereas isolate 2R was grown from a rectal VRE screening sample in a patient with complex intra-abdominal infection who had received tigecycline for only 3 days.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution or Etest (AB bioMérieux, Solna, Sweden) on Iso-Sensitest agar (Oxoid Ltd., Basingstoke,

UK) and were interpreted according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines [10].

2.3. Whole-genome sequencing

DNA was extracted using an Ultraclean® Microbial DNA Kit (Cambio Ltd., Cambridge, UK) for 1S, 1R, 3S and 3I according to the manufacturer's instructions and was quantified using the dsDNA HS Assay on a Qubit® fluorometer (Thermo Fisher Scientific, Renfrew, UK). Using 1 ng of input DNA, sequencing libraries were then generated using a Nextera XT Sample Preparation Kit (Illumina, Great Chesterford, UK) according to the manufacturer's recommendations. Sequencing was performed on a MiSeq™ Sequencing System (Illumina) using v2 reagents and generated 300 base (forward) and 200 base (reverse) paired-end reads. For isolates 2S and 2R, DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and was sequenced on a HiSeq™ 2000 instrument (Illumina) using 90 base paired-end reads at the Beijing Genomics Institute (Shenzhen, China).

2.4. Genome analysis

Raw reads were adapter and quality trimmed using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic). Reads were aligned against the *E. faecium* DO reference genome (accession no. **NC_017960.1**) using BWA-MEM (https://github.com/lh3/bwa), variants were called using VarScan2 with an allele frequency threshold of 80%, and the effect on coding sequences was predicted using SnpEff (http://snpeff.sourceforge.net). In addition, to confirm that complex

mutations in *rpsJ* did not result from mapping artefacts, a de novo assembly was performed on each sample using SPAdes 2.5.0 (http://bioinf.spbau.ru/spades) with default parameters. The location of the *rpsJ* sequence in each draft assembly was determined by BLASTX, and matching sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). To look for changes in gene content between pairs of isolates, the draft genome assemblies for each isolate were compared using the LS-BSR pipeline (https://github.com/jasonsahl/LS-BSR). The python script compare_BSR.py from the same package was used to compare the resistant or intermediate isolate against the sensitive isolate for each pair. Multilocus sequence typing (MLST) sequence types were determined in silico from the de novo assemblies using the python script mlst (https://github.com/Victorian-Bioinformatics-Consortium/mlst). The nucleotide sequences of the *E. faecium* isolates described in this work have been deposited at the European Nucleotide Archive under the study accession no. **PRJEB7380** (http://www.ebi.ac.uk/ena/data/view/PRJEB7380).

3. Results and discussion

The antibiotic susceptibility profiles revealed increased resistance to tigecycline in each of the three post-therapy isolates (Table 1). Each of the pairs showed different mutations in *rpsJ*, which encodes the S10 structural protein in the small 30S ribosomal subunit.

Comparison of genomes for pair 1 showed a codon deletion (ATC) and a non-synonymous transition (T>C) mutation. Pair 2 had a deletion of five codons (ATCCGTGCGACTCAT). Pair 3 had two non-synonymous transversion (C>A and

A>G) mutations (Fig. 1). All detected mutations were found in close proximity, corresponding to amino acid positions 52–60 in *E. coli* K-12.

Tigecycline resistance may result from changes to the regulation of efflux pumps, with other reported mechanisms including enzymatic degradation [3]. In the current study, we did not find evidence for such mutations (Supplementary Table S1). Analysis of gene content demonstrated that neither isolates 1R or 2R were found to contain any additional coding sequences compared with their respective sensitive counterparts. Isolate 3I, however, was found to contain an additional 73 coding sequences, the majority of which were located within a 48-kb region of the genome compared with the reference genome Aus004 (GenBank accession no. **CP003351**). We found no annotation evidence of *tetA* or *tetR* sequences or additional efflux pump-related sequences in this region. There was no commonality between lost or gained sequences between isolate pairs, making it unlikely that changes in gene content between isolates accounted for the phenotypic changes observed.

A mutation at codon 57 in *rpsJ* was previously described in a tigecycline-resistant *K. pneumoniae* [9]. It was hypothesised that this mutation, which is in close proximity to the tigecycline-binding site, could disturb the ribosomal structure in turn affecting the strong affinity of tigecycline to the ribosome. Our analysis indicates that amino acid substitutions and deletions contribute to increased tigecycline MICs in *E. faecium*. Notably, Cattoir et al. also suggested that intermediate resistance (MICs of 0.25–0.5 mg/L) in *E. faecium* is associated with a single nucleotide substitution in *rpsJ* resulting in a non-synonymous change in the conserved KYKD (to EYKD or KYKY)

motif at positions 57–60 [11]. This is important because the mutations are located in the same region of *rpsJ* as the mutations detected here.

However, the single amino acid substitutions detected in their study and ours do not appear sufficient to produce a fully tigecycline-resistant phenotype.

According to an X-ray crystal structure of the *Thermus thermophilus* ribosome, the tigecycline-binding site, which is located in the small ribosomal subunit, is composed almost exclusively of ribosomal RNA. All of the mutations that increase resistance to tigecycline occur in a relatively poorly conserved loop of S10, which appears to be involved in maintaining the structure of the tigecycline-binding site [12]. All of the mutations that potentially increase resistance substantially (1R and 2R) result in the deletion of one or multiple amino acids from this loop and would likely significantly distort the tigecycline-binding site. Isolate 1R contains a missense mutation resulting in a tyrosine to histidine substitution at position 58 (Supplementary Fig. S1). Interestingly, *T. thermophilus* naturally contains a histidine at this position [12], suggesting that the substitution could compensate for a destabilisation caused by the deletion of isoleucine-52.

Two isolates (2R and 3I) both had mutations in a coding sequence HMPREF0351_12599, annotated as a hypothetical protein. Protein domain searches of this product identified a YacP-like NYN (N4BP1, YacP-like nuclease) domain. YacP proteins are predicted to interact in a processome complex that catalyses the maturation of rRNA and tRNA, suggesting this mutation may affect the structure of the small subunit of the ribosome [13].

This study highlights the importance of screening for short insertions and deletions (indels) as well as single nucleotide polymorphisms when performing mutant analysis. The results demonstrate that full resistance is likely to instead depend on a further deletion, with or without additional amino acid substitutions. Indels are often not specifically looked for during routine bioinformatics analysis owing to historical difficulties in mapping reads containing indels when using Illumina short-read sequencing, and high indel rates associated with sequencing platforms such as 454, Ion Torrent and Pacific Biosciences [14,15]. These mutations will be important when constructing databases to perform in silico antibiotic resistance determination directly from whole-genome data.

In conclusion, we have shown that tigecycline resistance in *E. faecium* can arise through several alternative evolutionary paths in *rpsJ* following tigecycline therapy and that deletions in the coding sequence may be necessary for full resistance in this increasingly important nosocomial pathogen. The detection in this study of three distinct *rpsJ* genotypes, in addition to two additional genotypes found in a separate study, adds weight to the prediction that mutations in *rpsJ* are an important cause of tigecycline resistance in *E. faecium*.

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Ethical approval: Not required.

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Fig. 1. Mutations identified in the *rpsJ* gene potentially implicated in tigecycline resistance. The relevant amino acid sequence of the early wild-type (WT) isolates in comparison with the mutated isolates identified both in the intermediate (3I) and resistant (1R and 2R) strains from the current study and those from Cattoir et al. [11]. Sequences from *Escherichia coli* K-12 and *Thermus thermophilus* are shown for context. Codons are numbered according to annotations in *E. coli* K-12. The polymorphic region is indicated with a dotted box, and mutations are indicated by asterisks. MIC, minimum inhibitory concentration (in mg/L).**Table 1**

Antibiotic susceptibilities of *Enterococcus faecium* isolates

Isolate	MLST	MIC (mg/L) [interpretation [a]]					
		Ampicillin	Vancomycin	Teicoplanin	Linezolid	Daptomycin [b]	Tigecycline
1S	280	>8 [R]	>32 [R]	>32 [R]	2 [S]	2	0.125 [S]
1R	280	>8 [R]	>32 [R]	>32 [R]	>8 [R]	2	8 [R]
2S	203	>8 [R]	>32 [R]	16 [R]	2 [S]	4	0.125 [S]
2R	203	>8 [R]	>32 [R]	8 [R]	1 [S]	4	8 [R]
3S	–	>8 [R]	>32 [R]	>32 [R]	4 [S]	1	0.125 [S]
3I	–	>8 [R]	>32 [R]	>32 [R]	2 [S]	2	0.5 [I]

MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; R, resistant; S, susceptible.

[a] According to British Society for Antimicrobial Chemotherapy (BSAC) guidelines [10].

[b] No BSAC MIC breakpoint is available for this antibiotic for *E. faecium*.

	Sample	MIC	Alignment
			42 47 52 57 62 67 72
	T. thermophilus		LPTRVRRFTVIRGPFKHKDSREHFELRTHNR
	E. coli		LPTRKECFTVLISPHVNKDARDQYEIRTHLR
This study	WT	0.125	LPTERSLYTIIRATHKYKDSREQFEMRTHKR
	3I	0.5	LPTERSLYTIIRETRKYKDSREQFEMRTHKR
	2R	8	LPTERSLYTI-----KYKDSREQFEMRTHKR
	1R	8	LPTERSLYTI-RATHKHKDSREQFEMRTHKR
Cattoir *et al.*	AusTig/HMtig1+2	0.25	LPTERSLYTIIRATHKYKYSREQFEMRTHKR
	EF16	0.5	LPTERSLYTIIRATHEYKDSREQFEMRTHKR

Highlights

- Whole-genome sequencing permits de novo detection of antibiotic resistance in *Enterococcus faecium*.
- Reduced susceptibility to tigecycline is associated with substitutions in *rpsJ*.
- Resistance to tigecycline is associated with deletions in *rpsJ*.
- Protein modelling indicates that these deletions distort the tigecycline-binding site.